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Gestation phenology of the greater mouse-eared bat (*Myotis myotis*)

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Abstract. Reproductive strategies of hibernating insectivorous bats and reproduction phenology parameters can vary considerably as they will be influenced by ecological seasonality. While the exact timing of greater mouse-eared bat (*Myotis myotis*) reproduction is described through morphological assessment of dissected embryos, most recent studies have focused only on population or behavioural aspects of reproduction. In our study, non-invasive ultrasound examination and non-lethal blood sampling were used to assess basic reproduction parameters of *M. myotis* from two nursery colonies over two gestation periods with different weather conditions. We found that ovulation probably occurred before female emergence from the hibernacula between late March and mid-April, triggered by a short period of high ambient temperature lasting at least three days. On the other hand, the estimated date of blastocyst implantation only varied within four days despite weather conditions differing significantly over the two years. Though specific foetal growth velocity did not differ between localities, the date of parturition was influenced by shelter temperature in May (difference between localities) and average ambient temperature in April (difference between years). The progesterone level does not appear to be a suitable indicator of pregnancy stage as it changed significantly during pregnancy and did not correlate with foetus size. The total gestation period (from fertilisation to parturition) varied between 56 and 73 days in our study.

Key words: reproduction timing, temperate bat populations, ambient temperature, progesterone, ultrasound examination

Introduction

Temperate bats are subject to seasonal variations in their food supply, which means their active season is relatively short. As a result, female reproductive costs depend heavily on prevailing environmental conditions, food availability, and fat reserves (Willis 2017, Zukalová et al. 2022). One means of saving energy for reproduction is torpor; however,

any decrease in body temperature will slow the development of the foetus (Racey 1969, Racey & Swift 1981) and gestation parameters in general.

While reproduction of *Myotis myotis* has been studied intensively since the 1920s (e.g. Courrier 1922, Štěrba 1990), most modern studies have tended to concentrate on population (e.g. Zahn 1999, Zahn et al. 2006) or behavioural (e.g. Audet 1990, Bilo 1990) aspects of reproduction due to strict protection now

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in place for all bat species. Central European *M. myotis* females mainly form nursery colony roosts in attics between April and August, with adult males living separately in individual shelters (Bilo 1990, Zahn 1999). During the first half of June, the females give birth to a single pup, which is nursed for around three to four weeks and becomes self-sufficient at six weeks of age (Zahn 1999). The colonies then disperse around mid-August (Zahn et al. 2007).

Reproduction phenology differs significantly between southern and northern *M. myotis* populations, the majority of geographic differences being explained by either a) dissimilarities in the thermal characteristics of roosts (e.g. caves *vs.* buildings) or b) different conditions and lengths of hibernation and activity seasons (Rodriguez et al. 2003, Zahn et al. 2007). Compared to populations in southern regions, for example, those in northern latitudes are more influenced by poor weather conditions during gestation as the summers are shorter, cooler and wetter, threatening the survival of both juveniles and adult bats (Roer 1973, Zahn 1999). Furthermore, *M. myotis* females have been shown to reduce their flight activity when temperatures drop below 10 °C, with females of all reproductive states remaining in the maternity colony, and may resort to daily torpor when cool periods (temperature < 12 °C) persist for several days (Heidinger et al. 1989). During warmer temperatures, however, pregnant females will forage for longer than during the lactation and post-lactation periods (Audet 1990).

While *M. myotis* has a bicornuate uterus with both ovaries functional, blastocyst implantation only occurs in the right uterine horn (Paksuz 2011). A rare birth of twins has been recorded, with the two pups believed to be homozygotic (Sklenář 1960, Currie et al. 1988). Previously, specific *M. myotis* reproduction parameters were almost exclusively obtained through the morphological assessment of dissected embryos (Štěrba 1990, Paksuz et al. 2017), with few data from wild populations (Bilo 1990).

In this study, for the first time, we undertake progesterone measurements alongside ultrasound examinations of female *M. myotis* from two different maternity colonies over two consecutive gestation periods to evaluate basic reproduction parameters. We predict that the reproduction phenology of the two colonies will not differ as, first, ovulation should start after emergence from the hibernacula and should be triggered by warmer temperatures (Heideman 2000), and secondly, the bats should

emerge from hibernacula with similar microclimatic conditions during a relatively short period of a few days (cf. Řehák et al. 1994). However, the phenology of reproduction is predicted to differ between years with different weather conditions, which will mainly influence the length of the preimplantation period and, consequently, the date of parturition (cf. Zahn 1999). Finally, progesterone concentration will increase during gestation until parturition, reflecting the growth of the bat embryo (Currie et al. 1988).

Material and Methods

Two *M. myotis* nursery colonies were studied during the gestation period (May-June), both located in the attics of churches, with approximately 240 females sampled in 2020 and 2021 from the church in Otaslavice (OTA; 49.3848658 N, 17.0676067 E) and approximately 130 females sampled during 2020 from Lysice (LYS; 49.4538758 N, 16.5383114 E; colony did not return to the roost in 2021). Both localities were visited regularly during the expected period of births for visual inspection, with minimal disturbance to the roosting bats. During blood sampling and ultrasound examination, individuals were captured using a handheld net (ten bats per locality per day) and determined for the sex and reproduction stage. The sample size was limited by the authorisation obtained for the study; we had to balance colony disturbance against sample size. The bats were weighed using a spring scale, and the forearm was measured using electronic callipers. After measurement and identification, the bats were placed individually in cloth bags. Before release, each animal was supplied with water supplemented with glucose.

An automatic HOBO U23-001Pro v2 datalogger (HOBO data loggers, USA), placed in close proximity to the roosting bats, was used to monitor the ambient temperature inside each attic. Data for maximal ambient temperature and long-term average April temperature between 2002 and 2022 (climatic variables), measured at the Brno/Tuřany weather stations approximately 35 km from both localities, were provided by the Czech Hydrometeorological Institute, Brno.

Ultrasound monitoring of bat gestation and calculation of reproduction parameters

A portable LOGIQ V2 ultrasound device (GE Healthcare, USA) was used to monitor the progression of pregnancy in females at each capture site. For examination, the animal was first immobilised with the body kept horizontal with the ventral side up.

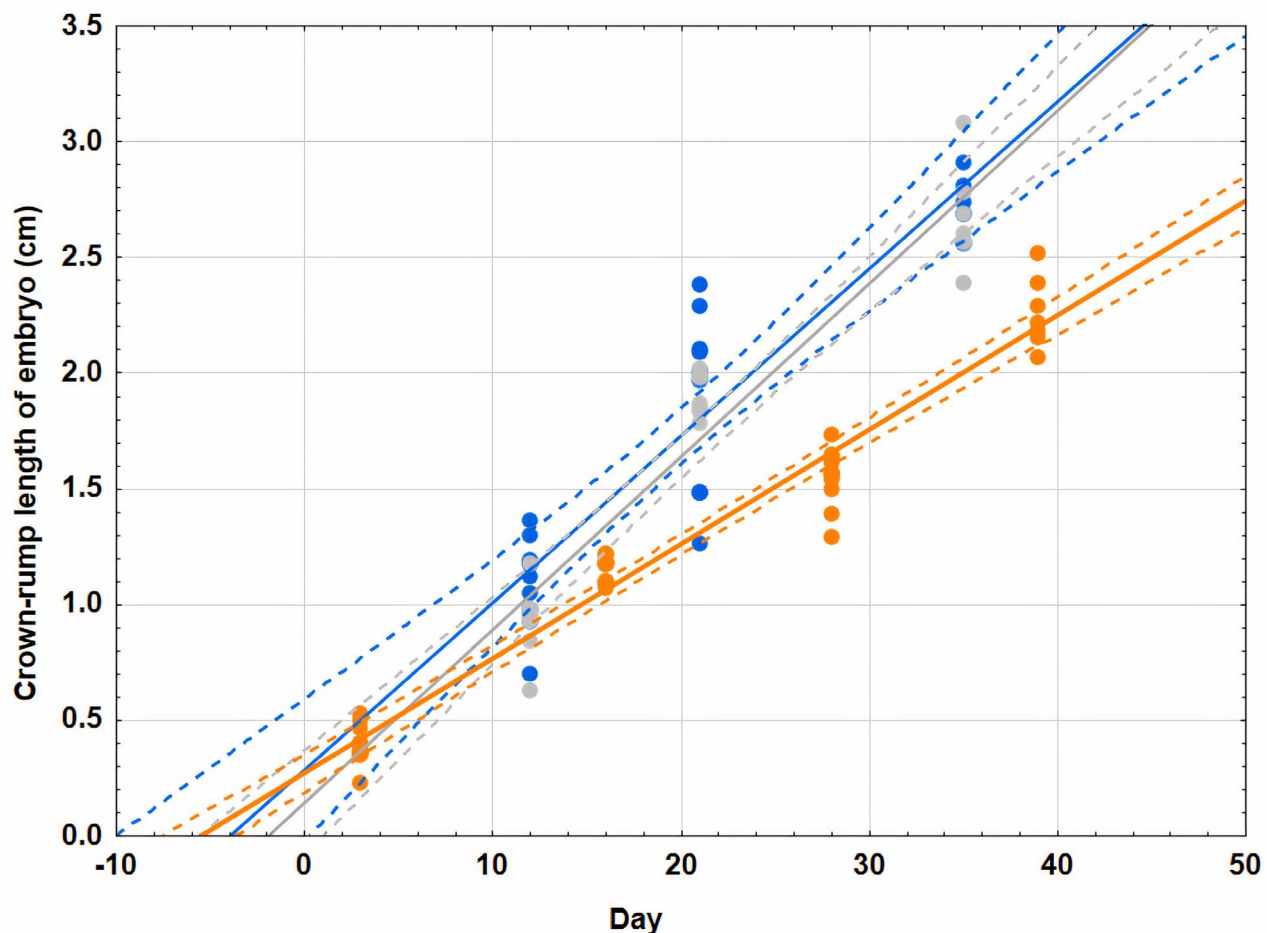


Fig. 1. Increase in crown-rump length over the gestation period (Day 1 – May 1st). Blue line – OTA 2020, grey line – LYS 2020, orange line – OTA 2021. Dashed line represents the confidence interval.

A neutral gel was then applied to the abdominal part, and the crown-rump length of the embryo was measured using a small 10 MHz microconvex 8CRS probe (GE Healthcare, USA). The bats were not marked as their recapture was almost impossible without significant disturbance to the nursery colonies. Consequently, population averages only are used for subsequent calculations.

Basic reproduction parameters, i.e. total gestation time (t_g) and specific foetal growth velocity (α), were calculated according to Huggett & Widas (1951), using the general formula $W^{1/3} = \alpha (t_g - t_o)$ to describe foetal development during the greater part of gestation up to full term, where W is the birth weight in grams and $t_g - t_o$ is the length of embryo development (i.e. time between implantation and parturition) in days. According to Austin & Bishop (1957), eggs only remain capable of normal fertilisation and development for less than 24 h in most mammals. Early evidence of deterioration or ageing appears soon after ovulation and is shown through a reduction in the rate of zona reaction and, particularly, in the blocking of

polyspermy. We assume, therefore, that ovulation and fertilisation occur over a very short period in bats, with sperm already present in the oviduct. Up to now, an arbitrary estimate of t_o (length of preimplantation period) has been used for temperate bats, i.e. $t_o \approx 0.3 t_g$ for species with gestation periods of 50 to 100 days. The period $t_g - t_o$ (length of embryo development, see above) was then calculated as the time between the point (date) where the x-intercept of a linear regression equation based on the crown-rump length of the embryo (i.e. date of blastocyst implantation; see Fig. 1) and the date of the median births observed or estimated in our colonies. We added six days to the date of the first birth (i.e. one day before the registration of the first juvenile in the colony) based on results from a colony of similar size under similar climatic conditions (Bilo 1990). As the date of first birth is a parameter tied to individual females, the calculation was used to obtain a median gestation length for the population, representing the time by which half of all juveniles had been born. The foetus weight at stage 24 (5.15 ± 0.89 g), published by Paksuz et al. (2017), was used as the birth weight

Table 1. Basic parameters of female *Myotis myotis* gestation at each study site.

Locality		Otaslavice		Lysice
Year		2020	2021	2020
Calculated date of ovulation		April 9 th	April 3 rd	April 11 th
Pre-implantation period	t_o	17	22	17
		$y = 0.286 + 0.0721 \cdot x$	$y = 0.2715 + 0.0494 \cdot x$	$y = 0.1429 + 0.0747 \cdot x$
		$r = 0.916; P < 0.001$	$r = 0.981; P < 0.001$	$r = 0.960; P < 0.001$
		$R^2 = 0.8389$	$R^2 = 0.9613$	$R^2 = 0.9217$
Implantation	n	24	37	24
	if $y = 0$	−4	−5	−2
	date	April 26 th	April 25 th	April 28 th
Length of embryo development	$t_g - t_o$	39	51	40
First birth		May 29 ^{th*}	June 9 th	June 1 ^{st*}
Median birth		June 4 th	June 15 ^{th*}	June 7 th
Total length of gestation	t_g	56	73	57
$W^{1/3}$		1.727		
Specific foetal growth velocity	α	0.0443	0.0339	0.0432

* Estimated values based on length of birth period published by Bilo (1990).

for *M. myotis*. At this point, the foetuses resemble new-borns, though their bodies show more intense pigmentation, and the claws of the forelimb and hindlimb are sharp and keratinised.

Blood sampling and progesterone analysis

Blood was only collected twice (May 12th and 21st) during the first year of the study, as described in Pikula et al. (2020). During the third control, births were already in progress and blood collection was prohibited by the local nature protection authorities. Briefly, the collection site was disinfected with alcohol, and approximately 2 µl of heparin was applied to the skin. The blood vessel was then punctured with a sterile needle, and the sample was collected using an automatic pipette with a sterile heparinised tip. This method provides up to 200 µl of blood, considered a safe volume for a mammal the size of a *M. myotis* bat (Morton et al. 1993). After blood collection, the puncture site was treated with Surgibond tissue glue (SMI AG, Belgium) to stop further bleeding. In the laboratory, plasma was separated by centrifugation and stored in a freezer at −20 °C until further analysis. Progesterone concentrations were determined using a Progesterone ELISA kit (Enzo Life Sciences Inc., USA), following the manufacturer's instructions.

Ethical approval

Bat capture complied with Czech Law No. 114/1992 on Nature and Landscape Protection and was based on permits issued by the Olomouc Regional Administration (Doc. No. KÚOK/55979/2021/OŽPZ/9063). All catching, handling and sampling of bats was carried out under a project licence approved by the Ethics Committee of the University of Veterinary Sciences, Brno (Doc. No. 45450/2019-MZE-18134). All methods were used in accordance with relevant international, national and institutional guidelines and regulations. All team members are authorised to handle wild bats according to Czech Certificate of Competency Nos. CZ01341 and CZ04344 (§17, Act No. 246/1992 Coll.).

Statistical analysis

The normal distribution of variables was tested using the Shapiro-Wilks test. A simple linear regression between the crown-rump length of the embryo and date was used to calculate the basic growth change parameters, while relationships between embryo length and date or progesterone concentration were assessed using Pearson's correlation coefficient. For all statistical analyses, May 1st was taken as day 1. Analysis of covariance (ANCOVA), with date as covariate, was used to compare the regression lines

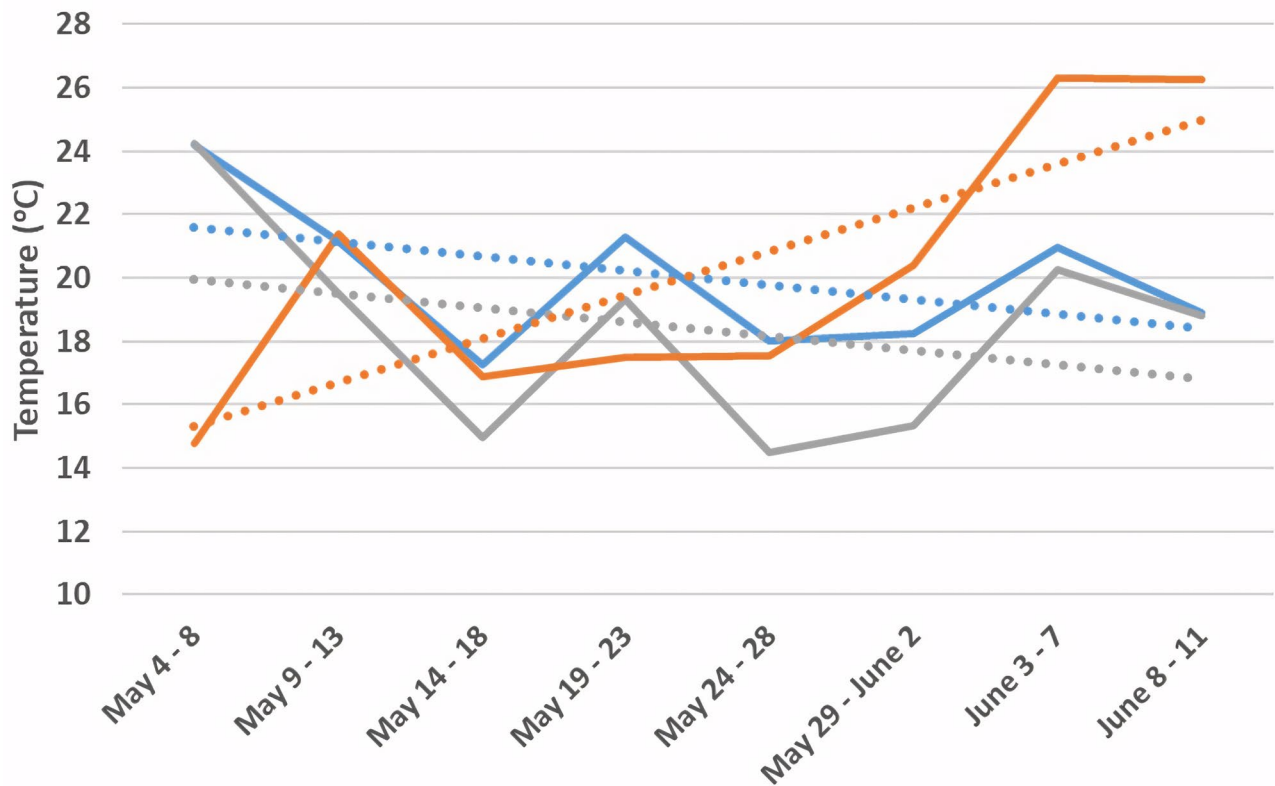


Fig. 2. Development of average shelter temperature in five-day periods during gestation (May-June). Blue line – OTA 2020, grey line – LYS 2020, orange line – OTA 2021. Dotted line represents the linear regression.

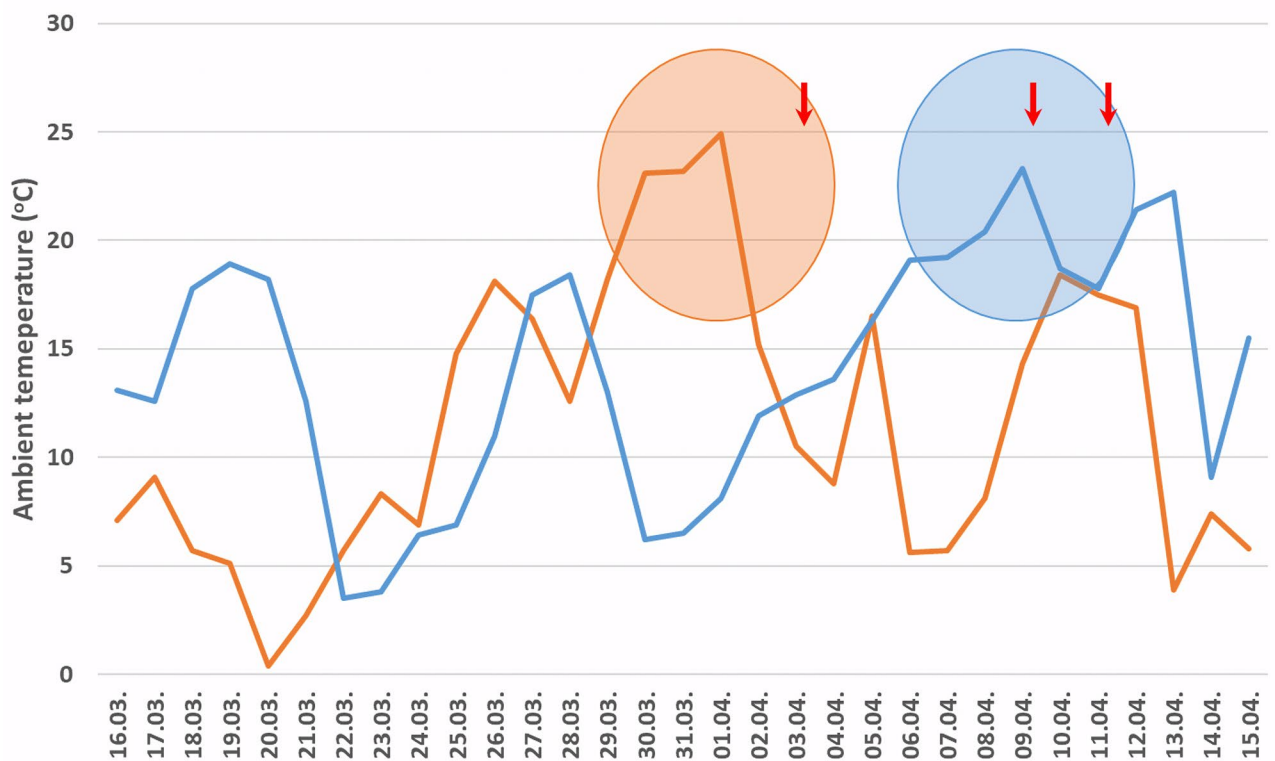


Fig. 3. Changes in maximal daily temperature over the late hibernation period (March 16th-April 15th). Blue line – 2020, orange line – 2021. Red arrows signify the estimated dates of ovulation based on data from Fig. 1.

obtained. Differences in progesterone concentration between the four measurements were compared using simple One-way ANOVA with *post-hoc* comparisons using the Fisher LSD test.

Results

Temporal parameters of gestation

The estimated date of blastocyst implantation varied within a four-day period from April 25th to 28th (Fig. 1, Table 1). Values from different years at Otaslavice were practically the same, despite weather conditions differing significantly during the emergence of bats from the hibernacula, with average monthly temperatures in April being 11.6 °C (0.81 °C above long-term average) and 7.62 °C (−3.17 °C below long-term average) in 2020 and 2021, respectively. The linear regression intercepts between embryo crown-rump length and date did not differ significantly between localities in 2020 (OTA 2020 *vs.* LYS 2020; $F_{1,45} = 1.497$, $P = 0.227$), but did between the two sampling years (OTA 2020 *vs.* OTA 2021; $F_{1,58} = 60.672$, $P < 0.001$).

The median birth date differed by a maximum of 11 days compared to the estimated day of ovulation,

which only differed by a maximum of eight days. Both of these parameters were influenced by shelter temperature, with the much colder first half of May 2021 prolonging bat gestation (Fig. 2). On the other hand, ovulation started much earlier in 2021, occurring shortly after a three-day period with maximal temperatures > 20 °C and minimal temperature > 5 °C between March 30th and April 1st (Fig. 3). A similar warm period occurred later in 2020, between April 7th and 13th, and this was again associated with onset of bat ovulation. These differences were also mirrored in the total length of gestation, which varied between 56 and 73 days, with a specific foetal growth velocity of around 0.04.

Changes in progesterone concentration

The progesterone concentration in a single non-reproducing young female was 35.58 ng/ml, and four other females had extremely low progesterone concentrations (between 64.38 and 77.53 ng/ml) compared to other females despite having developed an embryo; consequently, these five females were excluded from subsequent analysis. These statistical outliers may result from physiological factors not determined by our methods or through

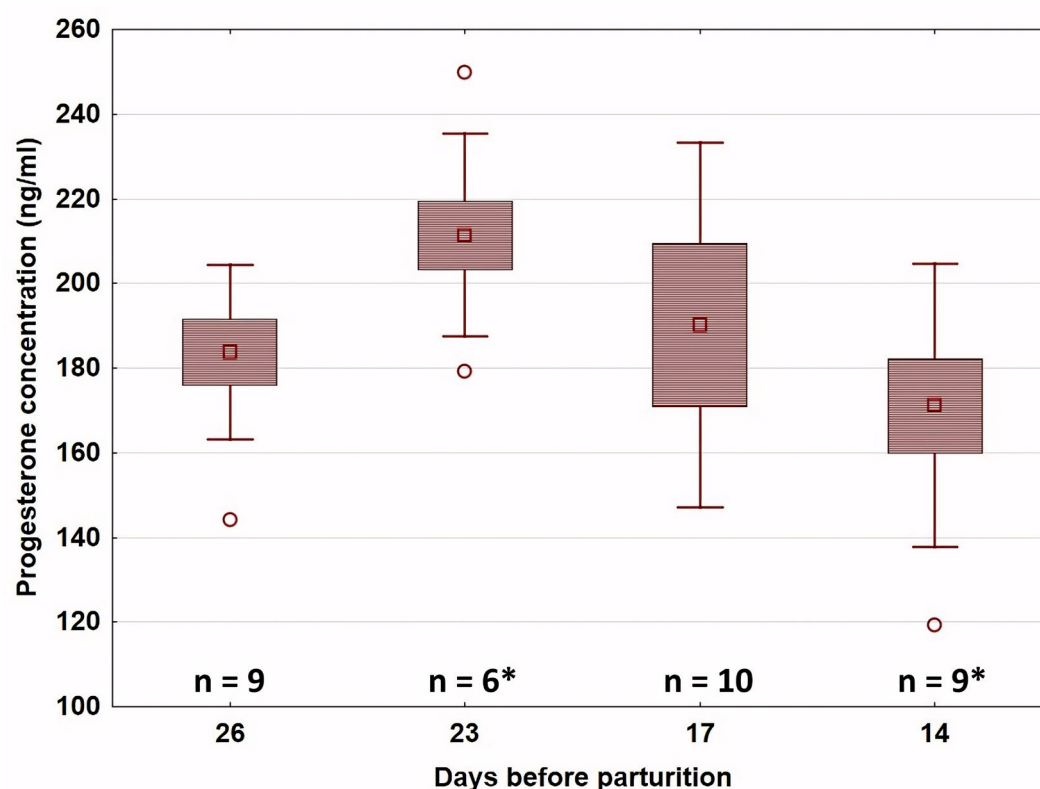


Fig. 4. Changes in blood progesterone concentration in *Myotis myotis* females during the 2020 gestation period. Samples are arranged according to the actual day of parturition (OTA 2020 – 26 and 17 days before median parturition, LYS 2020 – 23 and 14 days before median parturition). Square – mean, box – standard error, whiskers – SD, dots – outliers, n – sample size. Dates marked with asterisks differ significantly (LSD *post-hoc* comparison, $P = 0.009$).

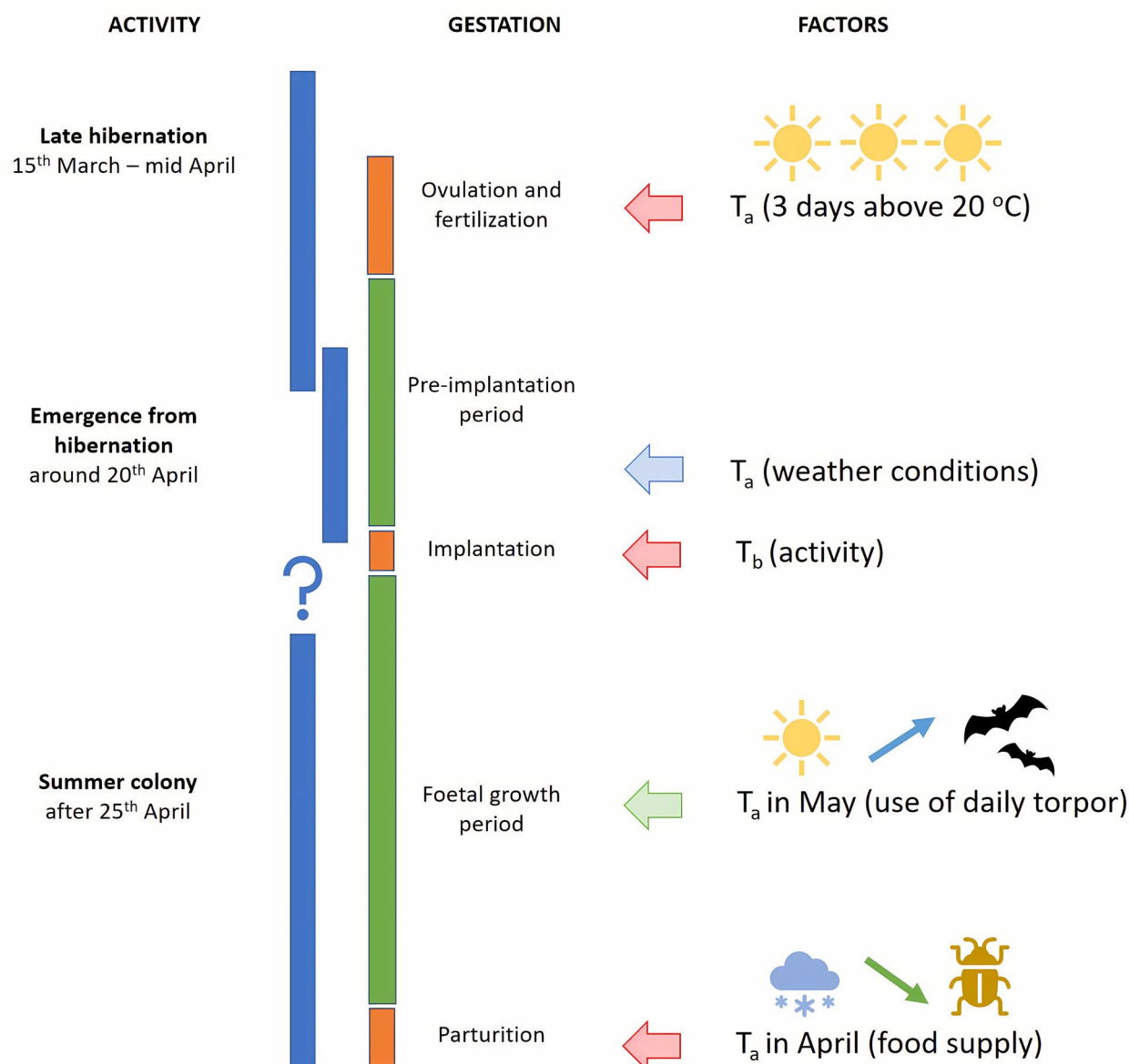


Fig. 5. Main factors influencing the course of gestation in *Myotis myotis* females. T_a – ambient temperature, T_b – body temperature, ? – unknown shelters during spring migration.

methodological errors in progesterone analysis. In all other cases, the linear regressions of embryo/foetus development did not differ between localities, thus data on progesterone concentrations were pooled for subsequent statistical analysis.

Progesterone concentration was uncorrelated with embryo size ($r = -0.270$, $P = 0.148$), increasing until three weeks before parturition (peak average value 211.39 ng/ml) and then decreasing (Fig. 4). While progesterone concentrations did not differ on different days before parturition ($F_{3, 26} = 2.805$, $P = 0.059$), a decrease in progesterone concentration during advanced gestation led to a significant difference between progesterone concentrations 23 days and 14 days before parturition (LSD *post-hoc* comparison, $P = 0.009$).

Discussion

As in most temperate bat species, female *M. myotis* utilise delayed ovulation. Ovulation occurs through a combination of external and internal factors and, in Central European latitudes, is timed to occur around mid-April, i.e. after emergence from the hibernacula (Heidemann 2000). However, our results suggest that, in *M. myotis*, ovulation, followed by immediate egg fertilisation, actually occurs at the hibernation site itself during the late hibernation period (cf. Zukal et al. 2005). It would appear that, under natural conditions, ovulation in this species is triggered by a minimum three-day period of maximum ambient temperatures > 20 °C and minimal temperature > 5 °C. Ambient temperatures not only affected the date of ovulation but also the duration of the



preimplantation period, even in bats hibernating in temperature-stable caves, as it is a critical climatic factor influencing both seasonal changes in activity and night-to-night bat flight activity patterns (Berková & Zúkal 2010). The total preimplantation period, from fertilisation to implantation, was at least 17 days, similar to the greater Asiatic yellow bat (*Scotophilus heathi*; 20–21 days; Pakrasi & Tiwari 2007) and Seba's short-tailed bat (*Carolia perspicilata*; 16–19 days; Cretekos et al. 2005). Implantation is coupled with the emergence of *M. myotis* from the hibernacula, occurring in the Moravian region during the second half of April (Řehák et al. 1994). Female *M. myotis* that hibernate in roosts with similar microclimatic conditions (e.g. Moravian Karst caves), blastocyst implantation has been shown to take place over a short four-day period. Furthermore, our data indicate that significantly different weather conditions outside of the roost in different years at Otaslavice did not influence the date of implantation. Such implantation synchronisation could occur after the females arrive at the maternity roost, possibly triggered by the reuniting of colony members after hibernation, during which interindividual transfer of progesterone may occur (Greville et al. 2017). This observation makes sense in light of the high birth synchrony previously recorded in bat maternity colonies (Bernard 1994), one of the most important mechanisms controlling colony cohesion.

As bats use daily torpor, gestation length is not fixed (Racey 1973, Racey & Swift 1981). Here, we confirm that ambient conditions also influence gestation length. While *M. myotis* is a typical gleaner, we confirmed that cold weather prolongs gestation in different years, similar to other temperate bat species, such as the greater horseshoe bat (*Rhinolophus ferrumequinum*; Ransome & McOwat 1994) and Daubenton's bat (*Myotis daubentonii*; Linton & MacDonald 2018). Ambient temperatures mainly influence the length of pregnancy in April and May. In *M. daubentonii*, a one-degree higher average temperature in April accelerates parturition by 2.8 days, even though this species can hunt at extremely low temperatures (-3.3°C ; Lučan et al. 2013). Likewise, female *R. ferrumequinum* give birth 18 days earlier on average when mean May temperatures increase by 2°C (Ransome & McOwat 1994). In our study, the length of pregnancy was also significantly influenced by average temperatures in April, with females giving birth 11 days earlier on average in 2020 when temperatures in April were approximately 3°C higher than average (average April temperatures

in 2021 were 3.17°C below the long-term average). Births should occur early enough for the young to learn to fly, hunt for food and accumulate fat reserves to survive winter (Zahn 1999, Rodríguez et al. 2003, Czenze et al. 2017).

Differences in the birth dates of juveniles in different geographical areas tend to be due to differences in temperature and availability of insects (Kunz 1974, Matthäus et al. 2023). For example, *M. myotis* births in the south Mediterranean mainly occur in early April, while most births in Germany, with its less stable weather, occur in June (Rodríguez et al. 2003). In this case, the bats from the southern Mediterranean formed nursery colonies in caves and thus experienced stable temperatures. In contrast, those from Germany used attics, which were far more influenced by outside temperatures and the intensity of solar radiation (Rodríguez et al. 2003). Studies comparing two different types of roost (i.e. buildings and caves) located in the same region, however, show bats with similar population characteristics and juvenile growth (Postawa & Gas 2009, Uhrin et al. 2010), with differences in the size of young between study years being explained by cool weather during pregnancy leading to a food shortage for mothers and a reduced growth rate for foetuses (Postawa & Gas 2009). Similarly, we believe that the differences in birth dates between years at Otaslavice were indirectly influenced by different ambient temperatures in April, resulting in a lowered food supply during 2021, whereas differences between localities within years were the result of differing roost temperature conditions at the beginning of May. In 2020, for example, juveniles were born three days earlier at the Otaslavice maternity colony than at the roost in Lysice, which was 2°C cooler on average.

In our study, the total gestation period for *M. myotis*, from fertilisation to parturition, varied between 56 and 73 days, consistent with earlier records (Herlant 1956, Sklenář 1960). Using data supplied by Paksuz et al. (2017), we assumed that the birth weight of neonate *M. myotis* was 5.15 g and calculated a specific foetal growth velocity (α) of 0.04 accordingly. Courier (1922) estimated a much higher value of up to 0.1 for *M. myotis*, while *Miniopterus* sp. (family Miniopteridae) had the lowest α value at 0.01, apparently due to delayed implantation and thus a significantly longer gestation period. However, a similar specific foetal growth velocity of 0.03 has been estimated for the black myotis (*Myotis nigricans*) and *Myotis lucifugus*, and 0.04 for the common noctule (*Nyctalus noctula*)

(Frazer & Huggett 1974). Consequently, our α value for *M. myotis* corresponds more closely to the values calculated for bats of the Vespertilionidae family than those of the Miniopteridae calculated by Courrier (1922). It should be noted, however, that this wide variation in α values could also be influenced by the different methods used for estimating total gestation length. Most authors now calculate gestation length as the number of days between the end of hibernation (cf. emergence from the hibernacula) and the date of birth (e.g. Harbusch & Racey 2006, Zukalová et al. 2022). Such calculation results in a significantly shorter estimated gestation period and a higher α value, and this must be considered in comparisons where the data are similar.

Progesterone is important as it ensures the endometrium has the appropriate thickness for implantation of the blastocyst and to maintain pregnancy. Additionally, it has pyrogenic properties that can help pregnant females maintain a higher body temperature (Racey 1973). In *M. lucifugus*, progesterone levels double for every 2 mm of foetal growth (Currie et al. 1988). We also observed increased progesterone levels during pregnancy in *M. myotis* females, though the increase was statistically non-significant. At the same time, we found no correlation between progesterone concentration and embryo/foetus size. As with *M. nigricans* (Beguelini et al. 2021) and *Pipistrellus pipistrellus* (Racey & Swift 1981), we observed an increase in progesterone concentration up to three weeks before parturition, after which levels decreased slightly, though these values were still much higher than those in non-reproductive females. While a substantial increase in plasma progesterone concentration also preceded the most rapid phase of foetal growth in *M. lucifugus*, there was no indication of any prepartum decrease in progesterone concentration (Buchanan & Younglai 1986, Currie et al. 1988). The reasons for these differences remain unknown. However, results have been equivocal, and the subject will require further study. When analysing the results obtained, it is also essential to consider the range of techniques used to measure plasma hormone levels in bats.

In summary, we conclude that the timing of gestation in *M. myotis* is both triggered and significantly influenced by ambient temperature, affecting all aspects of the total gestational period (Fig. 5). Our

data showed that ovulation can occur while the bat is still in the hibernaculum, having been triggered by a 3-5 day period of high ambient temperatures in the late phase of hibernation. On the other hand, implantation remained relatively stable over time, occurring shortly after the female emerged from hibernation. Once impregnated, specific foetal growth velocity was influenced directly by ambient temperature and indirectly by food supply (difference between years), though not by the temperature of the shelter or female energetics (no difference between localities within each year). In contrast, the timing of parturition was directly influenced by shelter temperature (difference between localities) and indirectly by average temperatures in April (difference between years), causing variation in the quantity of food available for pregnant females. Progesterone level was not a reliable indicator of pregnancy progress as concentrations increased up to three weeks before parturition but then decreased slightly, with no correlation to embryo/foetus size. For future research, it will be necessary to precisely define the total gestation period, as the two approaches used in Chiroptera, i.e. the time between emergence from hibernation (with assumed ovulation and implantation) and parturition and time between implantation and parturition, differ significantly.

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Author Contributions

J. Zukal, K. Zukalová and J. Pikula conceived and designed the study; J. Zukal, K. Zukalová and J. Pikula collected the material, with the support of V. Seidlová and P. Mrhálek; K. Zukalová and V. Seidlová performed the laboratory analysis; J. Zukal analysed the data; K. Zukalová and J. Zukal drafted the manuscript, to which all authors contributed with critical comments. All authors declare no conflict of interest.

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